

Short communication

Changes in macrophage and lymphocyte functions in guinea-pigs after different amounts of vitamin E ingestion

M. De la Fuente*, M. Carazo, R. Correa and M. Del Río

Department of Animal Physiology, Faculty of Biological Science, Complutense University of Madrid, Spain

(Received 22 April 1999 – Revised 11 November 1999 – Accepted 9 December 1999)

Vitamin E is the main biological lipid-soluble antioxidant and plays a crucial role in the maintenance of the immune system. In the present work, twenty-one guinea-pigs (3-weeks-old) were distributed into three groups, which during 5 weeks ingested different amounts of vitamin E (/kg diet): 15 mg (low vitamin E diet), 150 mg (medium vitamin E diet; control) or 1500 mg (high vitamin E diet). The function of lymphocytes and macrophages were then studied. In macrophages obtained from the peritoneum several steps of the phagocytic process (chemotaxis, ingestion and superoxide anion production) were assayed, as well as chemotaxis and proliferation of peritoneal and spleen lymphocytes. The results indicate that with respect to the medium vitamin E diet, low ingestion of vitamin E causes a decrease in chemotaxis and production of superoxide anion by macrophages and an increase in the phagocytic capacity. With the high vitamin E diet an increase in macrophage and lymphocyte chemotaxis, superoxide anion production and lymphoproliferative capacity, as well as a decrease in phagocytosis, were observed. Therefore, diet supplementation with higher than usual levels of vitamin E appears to be beneficial for the immune system.

Vitamin E: Leucocytes: Immune response: Guinea-pigs

Vitamin E is the most important lipid-soluble antioxidant present in body tissues and is considered the first line of defence against lipid peroxidation because of its quenching activity that protects cell membranes from free radical injury (Sies & Murphy, 1991). Determination of vitamin E requirements is controversial because high vitamin E levels may be necessary to prevent peroxidative damage. The current recommended dietary allowance for vitamin E in the USA is 10 mg/d, which falls into the range of acceptable intakes according to the COMA report (3.5–19.5 mg/d). However, although this vitamin E intake prevents the clinical deficiency syndrome, it fails to maintain optimal host defence especially in older subjects or in disease states (Beharka *et al.* 1997). Recent research provides evidence that a vitamin E intake much higher than the current recommendations can contribute to improved human health (Meydani *et al.* 1997; Weber *et al.* 1997). Results of several studies suggest that increased vitamin E intake is associated with a decreased risk of certain types of cancer (D'Avanzo *et al.* 1997; Peng *et al.* 1998) and CHD (Kushi *et al.* 1996). In fact, this antioxidant is involved in atherogenesis, inhibiting the proliferation of vascular smooth muscle cells (Azzi

et al. 1995) and reducing the susceptibility of LDL to oxidation (Simons *et al.* 1996). Moreover, on the basis of recent data it can be assured that vitamin E is safe and well tolerated at much higher than the recommended daily intakes and over long periods of time (Weber *et al.* 1997).

The important role of α -tocopherol in the defence against oxidative damage is especially relevant in leucocytes, because immune function and particularly phagocytic function is linked to the release of O_2 radicals that participate in the microbicidal activity of macrophages. Thus, the immune system has been shown to be more sensitive than other systems to antioxidant deficiencies in the diet (Meydani, 1998). Several authors have suggested that impaired host defence can act as a very early and sensitive marker of marginal deficiency of antioxidant micronutrients and thus, assessment of immune functions could serve as an important preventive diagnostic tool in the detection of marginal but functionally relevant micronutrient deficiencies (Schmidt, 1997).

Although it is known that the immune response is impaired when antioxidant vitamins are not present in the diet (Bendich, 1989), few reports deal with vitamin E effects

* Corresponding author: Professor M. De la Fuente, fax +341 394 4935, email mondelaf@eucmax.sim.ucm.es

on immune function. Stimulation of the lymphoproliferative response (Sakai & Moriguchi, 1997) and increased phagocytic activity (Moriguchi *et al.* 1990) have been shown. More recently, an inhibition of macrophage migration inhibitory factor was reported (Sakamoto *et al.* 1998). Our group has also carried out recent work on the effect of vitamin E on functions of murine leucocytes *in vitro* (Del Río *et al.* 1998) and on the immune response in a group of elderly women to whom a supplemented diet was administered (De la Fuente *et al.* 1998).

Considering the increasing interest on the beneficial effects of vitamin E intake, especially on the immune response, the aim of the present work was to study several immune functions in an animal model, namely guinea-pigs that were fed on diets containing three different amounts of vitamin E.

Animals and methods

Animals and diets

Dunkin-Hartley male guinea-pigs of 3 weeks of age were obtained from Iffa-Credo (Lyon, France). The animals were randomly divided into three experimental groups, each containing seven individuals, which received three diets differing in the vitamin E content for 5 weeks, i.e. low diet (15 mg vitamin E/kg basal diet), medium diet (150 mg vitamin E/kg basal diet) and high diet (1500 mg vitamin E/kg basal diet). The same basal diet (U.A.R., Perpignan, France) was administered to the three groups, which contained (g/kg): protein 185, fat 29, carbohydrate 469, mineral mix 84, vitamin mix 14, moisture 11, non-nutritive bulk 109. The content of minerals and vitamins was (/kg diet): P 8.6 g, Ca 10.6 g, K 12.0 g, Na 3.45 g, Mg 3.13 g, Mn 100 mg, Fe 320 mg, Cu 26 mg, Zn 85 mg, Co 1.61 mg, vitamin A 19 000 IU, vitamin D₃ 2031 IU, thiamine 22.5 mg, riboflavin 21 mg, pantothenic acid 1243 mg, pyridoxine 10.7 mg, menadione 55 mg, niacin 193 mg, folic acid 7.3 mg, biotin 0.275 mg, choline 1.74 g, myoinositol 250 mg, vitamin B₁₂ 0.054 mg, *p*-aminobenzoic acid 10 mg, vitamin C 660 mg. Guinea-pigs were housed in air-positive pressure animal cabinets (A 130 SP, Flufrance, Cachan, France) with an HEPA air-filter at the inlet (99.999 % for particles > 0.03 µm at the inlet).

Collection of leucocyte suspensions

The animals were killed by decapitation according to the guidelines of the European Community Council Directives 86/6091 EEC. Peritoneal suspensions were obtained from each animal following a method previously described (Ortega *et al.* 1992). Briefly, after intraperitoneal injection of 10 ml Hank's medium (Gibco Canada Ltd., Burlington, Ontario, Canada), the abdomen was massaged and peritoneal resident cells, containing macrophages and lymphocytes, were removed, allowing the recovery of 90–95 % of the injected volume. The macrophages, identified by morphology and non-specific esterase staining, and the lymphocytes were counted in Neubauer chambers and their concentrations were adjusted in the same medium at 5×10^5 cells/ml. The spleen was removed aseptically, freed of fat, minced with scissors and gently pressed through a mesh screen (Sigma,

St. Louis, MO, USA). The cell suspension was centrifuged in a gradient of Ficoll-Hypaque (Sigma) with a density of 1.070 g/ml. The material in the interface was resuspended in RPMI 1640 enriched with L-glutamine (Gibco Canada Ltd.) and supplemented with 10 % heat-inactivated fetal calf serum (Gibco Canada Ltd.) and gentamicin (100 µg/ml, Gibco Canada Ltd.), washed and the number of lymphocytes adjusted to 1×10^6 cells/ml. Cellular viability was routinely measured before and after each experiment by the Trypan-Blue exclusion test and was higher than 95 % in all experiments.

Chemotaxis, phagocytosis and superoxide production assays

These assays were carried out following methods previously described (De la Fuente *et al.* 1991). Chemotaxis was evaluated using chambers with two compartments separated by a filter (Millipore, Mildford, MA, USA) of 3 µm pore diameter. Aliquots of 300 µl of the peritoneal suspension were deposited in the upper compartment, and aliquots of 400 µl of the chemoattractant fMet-Leu-Phe (Sigma) at a concentration of 10^{-8} M in the lower compartment. The chambers were incubated for 3 h, the filters fixed and stained and the number of macrophages and lymphocytes in the lower face of the filter were counted and recorded as the chemotaxis index.

Phagocytosis of inert particles (latex beads (1.09 µm) diluted to 1 % in PBS) was carried out incubating aliquots of 200 µl of the peritoneal suspension in migratory inhibitory factor plates (Sterilin, Teddington, London, UK) for 30 min. The adhered monolayer was washed with PBS at 37°C, and 200 µl Hank's medium and latex (20 µl) were added. After 30 min of incubation, the plates were washed with PBS, fixed and stained, and the number of latex beads ingested per 100 macrophages were counted and recorded as phagocytosis index.

Superoxide production was determined by the Nitroblue Tetrazolium (Sigma) reduction test, based on an equimolecular reaction between Nitroblue Tetrazolium and superoxide anion (Bagasra *et al.* 1988). Aliquots of 250 µl of peritoneal suspension were mixed with 250 µl of Nitroblue Tetrazolium solution (1 mg/ml) and 50 µl latex beads were added to one sample set (stimulated samples) and 50 µl Hank's medium to the other set (non-stimulated samples). After 60 min of incubation in a bath at 37°C, the reaction was stopped, and following centrifugation, the supernatants were discarded and the reduced Nitroblue Tetrazolium was extracted with dioxan. The absorbance of supernatants was determined in a spectrophotometer at 525 nm.

Lymphocyte proliferative response assay

Proliferation of lymphocytes, spontaneous and induced by phytohaemagglutinin as mitogen, was determined in 72 h cultures. Aliquots of 200 µl of spleen lymphocyte suspension were seeded in ninety-six well flat-bottomed microtiter plates (Costar, Cambridge, MA, USA), and incubated in the absence or in the presence of phytohaemagglutinin (25 µg/ml) for 48 h at 37°C in an atmosphere of 5 % CO₂. To measure proliferation a BrdU labelling and detection

commercial kit (Roche Diagnostics, Basilea, Switzerland) was used. Briefly, it consisted of the addition to the culture medium of BrdU that is incorporated into freshly synthesized DNA. Following fixation of cells, cellular DNA is partially digested by nuclease treatment. A peroxidase-labelled antibody to BrdU is then added. At the final step, the peroxidase substrate is added, yielding a coloured reaction product as a result of peroxidase enzyme activity. The absorbance of the sample (measured at 405 nm) is directly correlated with the level of BrdU incorporated into cellular DNA.

Statistical analysis

The data are expressed as the mean and standard deviation of the values from seven animals, each value being the mean of duplicate assays. In the statistical study, the normality of the samples was confirmed by the Kolmogorov-Smirnov test and the homogeneity of variances by the Levene test. When the variances were homogeneous, the one-way ANOVA and Scheffe F test were used for the comparison of parametric samples. Conversely, when the variances were not homogeneous, the one-way ANOVA and Tamhane test were used.

Results

The results are shown in Table 1. Chemotaxis of peritoneal macrophages shows a significant increase with the diet high in vitamin E in comparison with the medium ($P < 0.01$) and low ($P < 0.001$) diets, and also a significant ($P < 0.01$) decrease with the low diet as compared with the medium one. With respect to the effects observed in lymphocytes, animals fed on the high vitamin E diet showed higher chemotaxis indexes ($P < 0.05$) than those fed on the low vitamin E diet. Phagocytosis of latex beads was significantly ($P < 0.001$) stimulated with the low vitamin E diet with respect to the others, while no significant difference was observed between the medium and high diets. The production of superoxide anion was significantly ($P < 0.001$)

reduced in guinea-pigs fed on the low vitamin E diet with respect to the others. Again no significant difference was observed between the medium and high diets.

A statistically significant increase was found in spontaneous lymphoproliferation with the high vitamin E diet with respect to the medium ($P < 0.01$) and low ($P < 0.001$) diets. The proliferation in response to the mitogen phytohaemagglutinin showed the same pattern with significant differences found for the high vitamin E diet with respect to the control ($P < 0.001$) and low ($P < 0.01$) diets.

Discussion

The effects of three different amounts of vitamin E in the diet of guinea-pigs have been studied. Animals in the low-vitamin-E-diet group ingested 0.6–0.75 mg/d, a dose very close to the minimum daily requirement for long-term maintenance of the growing guinea-pig, i.e. 1 mg/d (National Research Council, 1978). Animals in the medium-diet group ingested vitamin E in the normal range used for routine maintenance of guinea-pigs, 6-fold higher than the minimum daily requirement. Finally, the high-diet group was designed to clarify the effects of supplementing the diet with amounts of vitamin E 65-fold higher than the minimum daily requirement.

In the present work, an increase in the migration capacity of macrophages, which is an early step of the phagocytic process, was found with the high vitamin E diet. Recently it has been reported that vitamin E inhibits the secretion of macrophage migration inhibitory factor (Sakamoto *et al.* 1998), which provides a possible explanation for our results. The decreased chemotaxis of macrophages found in the guinea-pigs fed on the low vitamin E diet could indicate an oxidative state in these animals. Peritoneal macrophages from mice with oxidative stress caused by endotoxin have shown a decreased chemotaxis capacity (VÍctor *et al.* 1998), which seems to be due to a high production of migration inhibitory factor (Calandra & Bucala, 1997).

The phagocytic activity of macrophages was increased

Table 1. Effect of different vitamin E contents in the diet on variables of macrophages and lymphocytes function in guinea-pigs (Mean values and standard deviations for duplicate determination for seven guinea-pigs per group)

	Dietary vitamin E					
	Low (15 mg/kg diet)		Medium (150 mg/kg diet)		High (1500 mg/kg diet)	
	Mean	SD	Mean	SD	Mean	SD
Macrophage functions						
Chemotaxis index‡	108**	16	151	18	205**†††	23
Phagocytosis index§	827***	58	407	59	325†††	36
Non-stimulated O ₂ ⁻ production (A ₅₂₅)	0.017***	0.003	0.043	0.007	0.045†††	0.01
Stimulated O ₂ ⁻ production (A ₅₂₅)	0.028***	0.007	0.066	0.007	0.064†††	0.011
Lymphocyte functions						
Chemotaxis	50	6	59	8	62†	6
Spontaneous proliferation (A ₄₀₅)	0.172	0.008	0.179	0.028	0.231**†††	0.049
Proliferative response to mitogen (A ₄₀₅)	0.235	0.022	0.211	0.014	0.286***††	0.032

A, absorbance measured at the wavelength (nm) shown as subscript.

Mean values were significantly different from those of the medium vitamin E diet group (control): ** $P < 0.01$, *** $P < 0.001$.

Mean values were significantly different from those of low vitamin E diet group: † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$.

‡ The chemotaxis index was the number of cells in the lower face of the filter. For details of procedures, see p. 26.

§ The phagocytosis index was the number of latex beads ingested per 100 macrophages. For details of procedures, see p. 26.

for the cells from guinea-pigs that ingested the low vitamin E diet, a fact seen also by other authors in alveolar macrophages of rats (Moriguchi *et al.* 1990). Thus, these phagocytic cells exhibit in vitamin E deficient guinea-pigs a behaviour similar to that seen in mice with oxidative stress by endotoxic shock (V́ctor *et al.* 1998) or ageing (McArthur *et al.* 1998). With the diet high in vitamin E, a lower index of phagocytosis was obtained without significant differences between the medium and high vitamin E diets, which is in agreement with other authors (Hogan *et al.* 1992). Again, as in phagocytosis, we observed the same levels of superoxide anion production with the medium and high vitamin E diets, significantly higher than the corresponding values with low vitamin E doses. This supports the idea that the observed increment in phagocytosis found in response to the low vitamin E diet is not favourable from an immunological viewpoint since it does not result in an increase in microbicidal capacity.

The lymphoproliferative response is one of the most widely studied functions of lymphocytes. There are several reports on the effect of vitamin E supplementation on this pivotal immune activity, most of them showing a positive role of this antioxidant (Sakai & Moriguchi, 1997; McArthur, 1998). Accordingly, we also found an increased proliferation in response to phytohaemagglutinin mitogen in the animals that received the high dose of vitamin E. This action might be explained by a vitamin E induced decrement of prostaglandin E₂ production, which has been correlated with an increase in interleukin 2 production and concomitant raised proliferation (Beharka *et al.* 1997).

In conclusion, recent data support the idea that high ingestion of vitamin E is safe (Weber *et al.* 1997). In addition, our results indicate that a greater than recommended intake of vitamin E in the diet improves the immune response of adult guinea-pigs. Therefore, supplementation of the diet with higher amounts of this vitamin should be considered in order to improve human health.

Acknowledgements

We thank S. Burgos, B. De la Cuadra and M. D. Ferrández for their technical assistance. We also thank Dr A. Hernanz and Dr J. Miquel for their critical reading of the manuscript. This work was supported by a Grant from FISs (no. 97/2078).

References

- Azzi A, Boscoboinik D, Marilley D, Ozer NK, Stauble E & Tasinato A (1995) Vascular smooth muscle cell proliferation is controlled by α -tocopherol at the level of protein kinase C and of the transcriptional factors AP1. In *Oxidative Stress and Ageing* [R Cutler, L Packer, J Bertram and A Mori, editors]. Basel and Boston, MA; Birkhauser.
- Bagasra O, Howedy A & Kajdacsy-balla A (1988) Macrophage function in chronic experimental alcoholism. Modulation of surface receptors and phagocytosis. *Immunology* **65**, 405–409.
- Beharka AA, Wu DY, Han SN & Meydani SN (1997) Macrophage prostaglandin production contributes to the age-associated decrease in T cell function which is reversed by the dietary antioxidant vitamin E. *Mechanisms of Ageing and Development* **93**, 56–77.
- Bendich A (1989) Interaction between antioxidant vitamins C and E and their effect on immune responses. In *Handbook of Free Radicals and Antioxidants in Biomedicine II*, pp. 153–160 [J Miquel, AT Quintanilla and H Weber, editors]. Boca Raton, FL: CRC Press.
- Calandra T & Bucala R (1997) Macrophage migration inhibitory factor (MIF): a glucocorticoid counter-regulator within the immune system. *Critical Reviews of Immunology* **17**, 77–88.
- D'Avanzo B, Ron E, La Vecchia C, Franceschi S, Negri E & Ziegler R (1997) Selected micronutrient intake and thyroid carcinoma risk. *Cancer* **79**, 2186–2192.
- De la Fuente M, Del Río M, Ferrandez MD & Hernanz A (1991) Modulation of phagocytic function in murine peritoneal macrophages by bombesin, gastrin-releasing peptide and neuromedin C. *Immunology* **73**, 205–211.
- De la Fuente M, Ferrandez MD, Burgos MS, Soler A, Prieto A & Miquel J (1998) Immune function in aged women is improved by ingestion of vitamin C and E. *Canadian Journal of Pharmacology* **76**, 373–380.
- Del Río M, Ruedas G, Medina S, Víctor VM & De la Fuente M (1998) Improvement by several antioxidants of macrophage function in vitro. *Life Science* **63**, 871–881.
- Hogan JS, Weiss WP, Todhunter DA, Smith KL & Schoenberger PS (1992) Bovine neutrophil responses to parenteral vitamin E. *Journal of Dairy Science* **75**, 399–405.
- Kushi LH, Folsom AR, Prineas RJ, Mink PJ, Wu Y & Bostick RM (1996) Dietary antioxidant vitamins and death from coronary heart disease in postmenopausal women. *New England Journal of Medicine* **334**, 1156–1162.
- McArthur WP (1998) Effect of aging on immunocompetent and inflammatory cells. *Periodontology 2000* **16**, 53–79.
- Meydani M, Lipman RD, Han SN, Wu D, Beharka A, Martin KR, Bronson R, Cao G, Smith D & Meydani SN (1998) The effect of long-term dietary supplementation with antioxidants. *Annals of the New York Academy of Science* **854**, 352–360.
- Meydani SN, Meydani M, Blumberg JB, Leka LS, Siber G, Loszewski R, Thompson C, Pedrosa MC, Diamond RD & Stollar BD (1997) Vitamin E supplementation and in vivo immune response in healthy elderly subjects. A randomized controlled trial. *Journal of the American Medical Association* **277**, 1380–1386.
- Moriguchi S, Kobayashi N & Kishino Y (1990) High dietary intakes of vitamin E and cellular immune functions in rats. *Journal of Nutrition* **120**, 1096–1102.
- National Research Council (1978) Nutrient requirements of laboratory animals. Washington, DC: National Academy Press.
- Ortega E, Collazos ME, Barriga C & De la Fuente M (1992) Stimulation of the phagocytic function in guinea pig peritoneal macrophages by physical activity stress. *European Journal of Applied Physiology* **64**, 323–327.
- Peng YM, Peng YS, Childers JM, Hatch KD, Roe DJ, Lin Y & Lin P (1998) Concentrations of carotenoids, tocopherols and retinol in paired plasma and cervical tissues of patients with cervical cancer, precancer and noncancerous disease. *Cancer Epidemiology Biomarkers Prevention* **7**, 347–350.
- Sakai S & Moriguchi S (1997) Long-term feeding of high vitamin E diet improves the decreased mitogen response of rat splenic lymphocytes with aging. *Journal of Nutritional Sciences Vitaminology* **43**, 113–122.
- Sakamoto W, Nishihira J, Fujie K, Handa H, Ozaki M & Yukawa S (1998) Inhibition of macrophage migration inhibitory factor secretion from macrophages by vitamin E. *Biochimica et Biophysica Acta* **1404**, 427–434.
- Schmidt K (1997) Interaction of antioxidative micronutrients with host defense mechanisms. A critical review. *International Journal of Vitamin and Nutrition Research* **67**, 307–311.

- Sies H & Murphy ME (1991) Role of tocopherols in the protection of biological systems against oxidative damage. *Journal of Photochemistry and Photobiology B: Biology* **8**, 211–224.
- Simons LA, Von Konigsmark M & Balasubramaniam S (1996) What dose of vitamin E is required to reduce susceptibility of LDL to oxidation? *Australian and New Zealand Journal of Medicine* **26**, 496–503.
- Víctor VM, Miñano M, Guayerbas N, Del Río M, Medina S & De la Fuente M (1998) Effects of endotoxic shock in several functions of murine peritoneal macrophages. *Molecular and Cellular Biology* **189**, 25–31.
- Weber P, Bendich A & Machlin LJ (1997) Vitamin E and human health: rationale for determining recommended intake levels. *Nutrition* **13**, 450–460.